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14. ABSTRACT Post-traumatic epilepsy (PTE) is a major long-term complication of traumatic brain injuries (TBIs), which are often suffered by members of the Armed Forces. PTE usually develops within five years of a head injury, and it is often expressed as medically intractable hippocampal epilepsy. Although there are a variety of causes of traumatic epilepsy, the resulting chronic neurological condition is characterized by common features, including recurrent spontaneous seizures, neuronal damage, mesial temporal lobe epilepsy (MTLE) in ~30% of patients, and resistance to available anticonvulsant drugs. Therefore, it is of critical importance to develop novel models to study post-traumatic epilepsy in order to facilitate the discovery of new treatments.					
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“New Treatments for Drug-Resistant Epilepsy that Target Presynaptic Transmitter Release”.

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INTRODUCTION: Post-traumatic epilepsy (PTE) is a major long-term complication of traumatic brain injuries (TBIs), which are often suffered by members of the Armed Forces. PTE usually develops within five years of a head injury, and it is often expressed as medically intractable hippocampal epilepsy. Although there are a variety of causes of traumatic epilepsy, the resulting chronic neurological condition is characterized by common features, including recurrent spontaneous seizures, neuronal damage, mesial temporal lobe epilepsy (MTLE) in ~30% of patients, and resistance to available anticonvulsant drugs. Therefore, it is of critical importance to develop novel models to study post-traumatic epilepsy in order to facilitate the discovery of new treatments. **Background:** during epileptogenesis, seizure-related functional and structural reorganization of neuronal circuits leads to both hyperexcitability of glutamatergic neurons and defective inhibition. While many postsynaptic alterations have been demonstrated, there is surprisingly little known concerning dysfunction of presynaptic transmitter release machinery in epilepsy. The recent successful introduction of the antiepileptic drug levetiracetam (LEV), which acts on presynaptic molecular targets, suggests that controlling dysregulation of presynaptic function could be a promising new therapeutic target for the treatment of unresponsive epilepsies. While LEV has been shown to bind to both the synaptic vesicle protein SV2a and N-type Ca^{2+} channels, its precise mechanism of action is not understood. Recent studies have found that severe seizures can down-regulate the expression of both SV2a and the group II metabotropic glutamate (mGluRII) autoreceptor (*i.e.* mGluR2) that normally control glutamate release from presynaptic terminals.

HYPOTHESIS AND OBJECTIVES: During periods of intense neuronal activity such as seizures, a larger pool of vesicles could result in more glutamate being released and long-lasting aberrant excitation. We propose to explore the effects of seizures on transmitter release and the presynaptic action of AEDs on these changes. We will use electrophysiology and multiphoton confocal microscopy. Preliminary data indicate that SE induces long-lasting potentiation of synaptic vesicle release in epileptic rats. We hypothesize that successful AED treatment might prevent or reverse these seizure-induced molecular deficiencies (reduction of N-type VGCC, mGluR II and SV2a expression), and be antiepileptogenic as well. Our **central hypothesis** is that pharmacological regulation of glutamate transmitter release at presynaptic sites will be an effective, novel therapeutic strategy to ameliorate epileptogenesis and excessive synaptic excitation in epilepsy.

The **long-term objectives** of this collaborative proposal are to: (1) investigate whether down-regulation of SV2a is responsible for reducing the anticonvulsant efficacy of LEV (this

phenomenon is known as tolerance and has limited the use of LEV in the treatment of epilepsy), (2) identify the most effective AEDs which modulate presynaptic glutamate release, and (3) determine the presynaptic mechanism of action of the new AED LEV to modulate vesicular release properties. *We predict that pharmacological regulation of glutamate transmitter release at presynaptic sites will be an effective, novel therapeutic strategy to treat many cases of drug-resistant epilepsy, especially epileptogenesis following traumatic brain injury.* In Year 3 of this proposal, we developed experiments to accomplish the **specific aim 3** (below).

Benefits of the Proposed Project: Experiments in this proposal will provide a better understanding on the presynaptic mechanism of action of classical and new AEDs with direct potential for improving the pharmacological management of epilepsy.

Specific Aim 3: *Assess whether down-regulation of mGluR2 and SV2a at mossy fiber terminals can be prevented by chronic treatment with presynaptic acting antiepileptic drugs.*

Working hypothesis: Reduced expression of SV2A and mGluR2 results in deficient mGluR2-mediated suppression of presynaptic release and reduced anticonvulsant efficacy of LEV. To evaluate the role of altered mGluR2 and SV2a expression epilepsy, we evaluated whether treatment with levetiracetam reduce transmitter release as evaluated using SpH transgenic mice and confocal laser scanning microscopy. In addition, we investigated whether changes in transmitter release (functional data) are associated with changes in expression of mGluR2 and SV2A and related proteins SV2B and SV2C.

BODY: Description of research accomplishments toward accomplishing the aims.

1. Imaging presynaptic transmitter release using Leica Laser Scanning Confocal Microscope for functional imaging and physiology. This microscope was used for functional imaging including analysis of stimulus-evoked changes in fluorescence of SynaptopHlourin (SpH) in transgenic mice. Experiments to tackle Specific Aim 3.1 (below) were performed using this technique.

2. Development of the pilocarpine model of epilepsy in mice and rats

Model of epilepsy in SPH mice: During the second year, we continued developing and improving the pilocarpine model of epilepsy in mice and rats at both institutions. We addressed a concern in relation to variability in the seizure patterns in transgenic SpH mice obtained from Jackson laboratories, Inc specifically B6.CBA-Tg(Thy1-spH)21Vnmu/J (Stock Number: 014651) when compared to mice animals originally provided by Dr. Venkatesh N. Murphy (Harvard University) to our collaborator Dr. Stanton (New York Medical College).

During year 1, we acquired SpH transgenic mice from Jackson Laboratories to establish a newly refreshed colony and develop the pilocarpine model of epilepsy in parallel with Dr. Stanton's laboratories. For our surprise, animals obtained from Jackson laboratories, Inc exhibited an increased resistance to enter *status epilepticus* in contrast to colonies established at Dr. Stanton's laboratory. These negative results contrasted with our

SV2A, SV2B, and SV2C. After animals were sacrificed, blood samples were collected (at endpoint) to measure plasmatic levels of levetiracetam using high-pressure liquid chromatography (HPLC) in treated versus non-treated groups.

Brief description of methods for hippocampal slice preparation: Brains from ~60 days of status epileptic or control SpH mice were used for all experiments, and procedures were approved by The University of Texas at Brownsville Institutional Animal Care and Use Committee (Protocol 2011-001-IACU). After anesthesia, rats were decapitated and their brains were quickly removed and submerged in ~0° C artificial cerebral spinal fluid (ACSF) solution containing (in mM) 124 NaCl, 3 KCl, 2 CaCl₂, 1.3 MgSO₄, 1.25 NaH₂PO₄, 25 NaHCO₃ and 10 glucose. For preparation the hippocampal slices, the whole brain excluding the olfactory bulbs was rapidly removed after decapitation and immediately in cooled in oxygenated ice-cold ACSF. Horizontal hippocampal slices were cut at 350 µm using Leica Vibratome. The experiments were performed in slices from control and SpH mice treated or non-treated with levetiracetam.

Analysis of presynaptic vesicular release in slices from SpH mice using laser scanning confocal microscopy: Imaging of SpH-positive mossy fiber boutons in brain slices was performed using a slightly modified protocol as the one previously described in our manuscript published in Brain². Briefly, changes in SpH fluorescence upon release were visualized with a spectral Leica TCS SPE laser-scanning confocal in a Leica DM6000 FS microscope with the objective ×63/0.90 NA water immersion infrared objective lens and a multispectral Leica confocal laser scan unit. Briefly, the light source was a solid state laser (488nm/10 mW). Epifluorescence from SpH-positive mossy fiber boutons was detected with photomultiplier tubes of the confocal laser scan head and emission spectral window was optimized for signal over background. A 565-nm dichroic mirror (Chroma Technology) separated green and red fluorescence to eliminate transmitted or reflected excitation light. Although there were no signs of photodamage, we used the lowest intensity needed for adequate signal-to-noise ratios. To detect position of the slices in the recording chamber, images were obtained at low magnification (x5 objective) using DIC, a CCD camera and video monitor. After positioning the slices in the proper position with image field over the *stratum lucidum* (area of mossy fiber pathway), setting were changed to epifluorescence in the microscope to detect level of fluorescent and confirm that the transgenic animal indeed express adequate levels of SpH fluorescence for the experiments. For SpH fluorescence experiments, slices were perfused with 25 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris) and 50 µM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5; Tocris). A 600 stimulus 20-Hz train in the stratum lucidum evoked SpH fluorescence increases (imaged within 100 µm from stimulating electrode) in the proximal region (the first 100 µm) of CA3 apical dendrites and images were acquired every 30 s. As previously reported, we use SpH as a pH-sensitive indicator of vesicular release in brain slices bathed in artificial CSF (containing 25 µM CNQX and 50 µM D-AP5 to prevent synaptically driven action potentials and epileptiform activity) in our recording chamber with pH maintained at or ~7.4². To calculate half-time of post-stimulus decay of SpH peak fluorescence intensity, ($t_{1/2}$) was calculated for each punctum by single exponential fits to destaining curves using the equation: $y_0 + A_1 e^{(-x/t_1)}$, with t_1 the decay constant. Data were analyzed with OriginPro.

Results: Four different groups of animals were analyzed to determine whether chronic treatment with levetiracetam *in vivo* may reduce presynaptic vesicular transmitter release in the mossy fiber pathway from granule cells in hippocampus of pilocarpine-treated epileptic SPH transgenic mice. Changes of SpH fluorescence were induced by a train of 600 action potentials delivered to the mossy fiber pathway using a bipolar stimulating electrode. Images of stimuli-induced SpH fluorescence changes were detected using laser scanning confocal microscopy (see above). We first compared normalized peak fluorescence changes after stimuli in control versus pilocarpine-treated (suffering status epilepticus) group of animals that were injected with saline instead of levetiracetam for 1 month (**Figure 1A**). As previously reported we detected a significant 4.4% increase in normalized peak fluorescence in status epilepticus (SE) group ($F_{\text{peak}} = 119.25 \pm 2.13\%$, $n = 4$, 106 boutons, 7 slices) when compared to saline-injected control group ($F_{\text{peak}} = 114.18 \pm 1.19\%$, $n = 5$, 112 boutons, 10 slices) (**Figure A, b3**). These data is consistent with our previous findings that status epilepticus induce an abnormal increase in presynaptic vesicular release as measured by SpH fluorescence changes in transgenic mice². To determine if chronic treatment with levetiracetam can ameliorate or prevent such increase in presynaptic vesicular release, we treated pilocarpine-injected animals with levetiracetam (i.p, dose: 100 $\mu\text{g/kg}$) during one month immediately following *status epilepticus* and compared this group to control animals treated with similar drug administration protocol simultaneously. Analysis demonstrated no significant differences in of normalized peak fluorescence changes after stimulation of mossy fibers between these groups indicating that chronic treatment with levetiracetam corrected presynaptic function abnormalities previously detected after *status epilepticus* (**Figure 1B**).

Plasmatic concentration of levetiracetam after treatment was assessed by high-pressure chromatography (HPLC) assays. No significant changes were detected between controls ($57.1 \pm \mu\text{g/ml}$) versus and *status epilepticus* groups ($47.9 \pm 1.5 \mu\text{g/ml}$, Student t-test, $p > 0.05$).

Conclusions: Chronic treatment with levetiracetam after *status epilepticus* can revert abnormalities (*i.e.* abnormally enhanced vesicular release) in presynaptic function of glutamatergic (excitatory pathways (*i.e.* mossy fibers) that may be responsible for epileptogenesis and hyperexcitability in mesial temporal lobe epilepsy. Accordingly, reduction in abnormally enhanced presynaptic vesicular release is possibly the main mechanisms of action of levetiracetam. Data in subsequent experiments indicate that this effect may be mediated by levetiracetam-induced up-regulation of its own target SV2A which is pathologically down-regulated after *status epilepticus*. However, further experiments are necessary to elucidate how levetiracetam reduce presynaptic release in epileptic synapses.

Figure 1

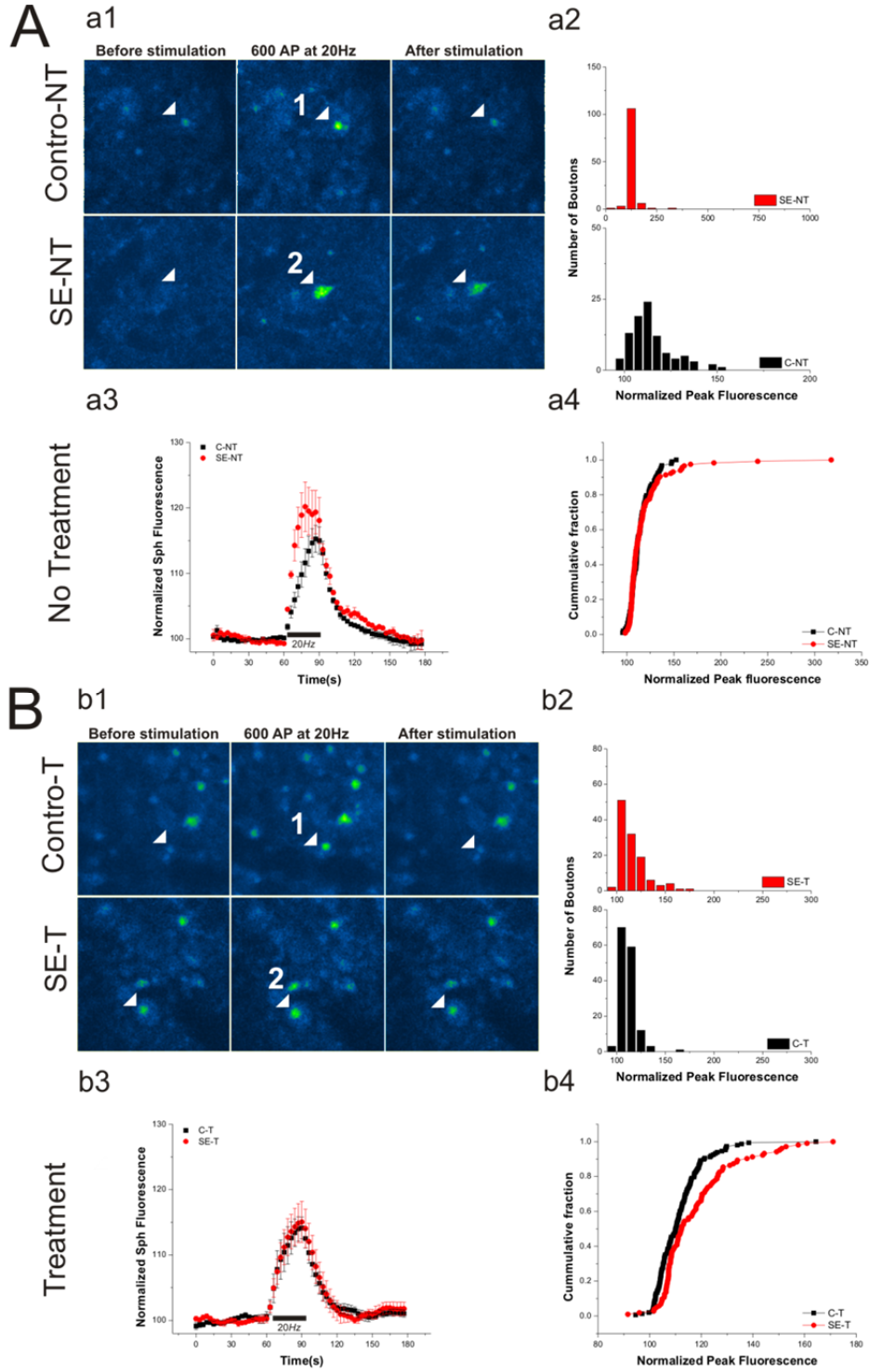


Figure 1. Effect of levetiracetam on the pilocarpine model to induced *status epilepticus* (SE) in SpH transgenic mice. **A.** *Stimuli-induced changes in presynaptic vesicular release at mossy fiber boutons in control and pilocarpine-treated SpH transgenic mice (no-treatment).* **a1.** Representative time-lapsed confocal images from control and post-*status epilepticus* SpH-expressing mossy fiber boutons MFBs in the proximal apical dendritic region of field CA3 in hippocampal slices of saline-treated control versus mouse one month after suffering SE treated with saline vehicle (lower row). First column: baseline imaging, second column: imaging during 600 action potential train stimulation, last column: recover of fluorescence changes 10 sec after end of stimulation. Notice larger increase in fluorescence changes after SE (compared arrowhead 1 to 2). **a2.** Frequency distribution histogram of normalized peak SpH fluorescence for all mossy fiber boutons after stimulation in control (black) compared to *status epilepticus* (SE) group (red). Notice a change in the distribution pattern, specifically a large group of mossy fiber boutons that release more than 200% increase of baseline after *status epilepticus* while peak fluorescence changes in mossy fiber boutons from control saline-treated animals follow a normal distribution. **a3.** Normalized, evoked SpH fluorescence increases in response to a 600 pulse/20 Hz mossy fibre stimulus train, in MFBs from control (filled black circles, $F_{\text{peak}} = 114.18 \pm 1.19\%$, $n = 5$, boutons=112, 10 slices) versus post-*status epilepticus* (open red circles, $F_{\text{peak}} = 119.25 \pm 2.13\%$, $n = 4$, boutons=106, 7 slices). F_{peak} was significantly increased in post-*status epilepticus* slices ($P < 0.05$, Student's *t*-test; all values mean \pm SEM). **a4.** Cumulative histogram distribution of normalized peak fluorescence changes in control (black) versus SE group (red) showing a left shift and a significant difference towards larger fluorescence peak changes (more release) in slices from post-*status epilepticus* animals ($D = 0.23$, $Z = 0.0305$, $p < 0.00197$, statistical comparisons using Kolmogorov-Smirnov test). **B.** *Chronic treatment with Levetiracetam (30 days period) after pilocarpine-induced status epilepticus normalized abnormally enhanced vesicular release at mossy fiber boutons.* **b1.** Time-lapsed images from representative experiments in slices from levetiracetam-treated control and epileptic SpH transgenic mice. Solid arrows indicate puncta corresponding to SpH-positive mossy fiber bouton that showed activity-dependent fluorescence changes during a 600 pulse/20 Hz stimulus train. **a2.** Frequency distribution histogram of normalized peak fluorescence for pooled mossy fiber boutons in control (black) versus pilocarpine-treated mice (red) chronically treated with levetiracetam after *status epilepticus*. **b3.** Representative time course of normalized, evoked SpH fluorescence increases in response to a 600 pulse/20 Hz mossy fibre stimulus train, in MFBs from control (filled black circles, $F_{\text{peak}} = 115.09 \pm 0.67\%$, $n = 3$, boutons=148, 9 slices) versus post-*status epilepticus* (open red circles, $F_{\text{peak}} = 116.25 \pm 1.27\%$, $n = 3$, boutons=106, 6 slices). F_{peak} was not significantly changed in levetiracetam-treated post-*status epilepticus* animals ($P > 0.05$, Student's *t*-test; all values mean \pm SEM). **b4.** Cumulative frequency histogram of normalized peak SpH fluorescence between the levetiracetam-treated control and SE group (red). Kolmogorov-Smirnov two-sample test indicated a significant difference between both groups ($t = 2.7$, $DF = 18$, $p = 0.014$).

3.2. *Status epilepticus*-induced abnormalities in presynaptic protein expression: effect of chronic treatment in vivo with levetiracetam.

In previous studies, we have detected a down-regulation of both metabotropic glutamate receptor type 2 (mGluR2) and SV2A presynaptic after pilocarpine-induced *status epilepticus*^{6,7}. This is particularly relevant because mGluR2 is a presynaptic regulator of transmitter release reducing excessive glutamate release during conditions of hyperexcitability (*i.e.* epilepsy)⁶⁻¹¹. SV2A is the molecular target of the antiepileptic drug levetiracetam. Previous studies in experimental animals of epilepsy and in patients with epilepsy have indicated that expression of SV2A is also chronically decreased in the epileptic tissue¹²⁻¹⁴. In this project we investigated how *status epilepticus* can alter expression of synaptic vesicle proteins (SV2A, Sv2B, and SV2C) (3.2a) and whether chronic treatment with levetiracetam can reverse those changes after *status epilepticus* to restore the presynaptic release machinery responsible for epileptogenesis (3.2b).

3.2a *Status epilepticus*-induced abnormalities in presynaptic protein expression.

Using immunohistochemistry, western blotting and real-time quantitative PCR (qPCR) analysis we have previously demonstrated *status-epilepticus*-induced abnormalities and changes in presynaptic protein expression of SV2A, SV2B and SV2C in Sprague Daley rats (2012-2013 Progress Report). We then tested whether similar abnormalities can be reproduced in mice considering that mice were used for the levetiracetam treatment in vivo due to 2 reasons 1) *data on presynaptic function (status epilepticus-induced abnormal presynaptic release of vesicles)* and 2) *rats are a larger species requiring larger amounts of drugs per kg of body weight (levetiracetam is a relatively expensive drug)*.

Immunohistochemical analysis of protein expression of SV2A, SV2B, SV2C. Multiple immunofluorescent staining was performed in histological sections from control and epileptic mice sacrificed 1-2 months after *status epilepticus*. We used SpH-positive (transgenic) and negative (no SpH) mice. SV2A protein expression was detected using polyclonal antibodies against SV2A (1:1000) from Synaptic System, Inc (Germany) with green channel secondary detection using AlexaFluo488 antibodies (Life Technologies). To identify the mossy fibers, antibodies against vesicular glutamate transporter type 1 (vGluT1) (1:2000, Millipore Corp, USA) were visualized with red-shifted AlexaFluo594 secondary antibodies. Neurons were labeled with anti-NeuN antibody visualized using AlexaFluo647 secondary antibodies to determine relative position of main hippocampal neuronal layers (pyramidal cell layer and granule cell layers). Imaging was performed using laser scanning confocal microscope Fluoview System in the IX81 inverted microscope (Olympus).

Results: Immunohistochemistry data revealed a down-regulation of SV2A in the chronic phase of the pilocarpine model of epilepsy in SpH mice (animals sacrificed after 2 months of *status epilepticus*) (**Figure 2**) while no qualitative changes were observed in the expression of SV2B (**Figure 3**) non-SpH mice. In addition, up-regulation of SV2C was noticeable in chronically epileptic mice, specifically, SV2C expression (intensity of fluorescent signal) was increased in the mossy fiber pathway, and in the outer layers of dentate gyrus consistent with abnormally sprouted mossy fiber bouton synapse onto granule cell dendrites forming a “recurrent excitatory pathway” the landmark of altered synaptic reorganization in this experimental model of temporal lobe epilepsy (**Figure 4**).

Figure 2

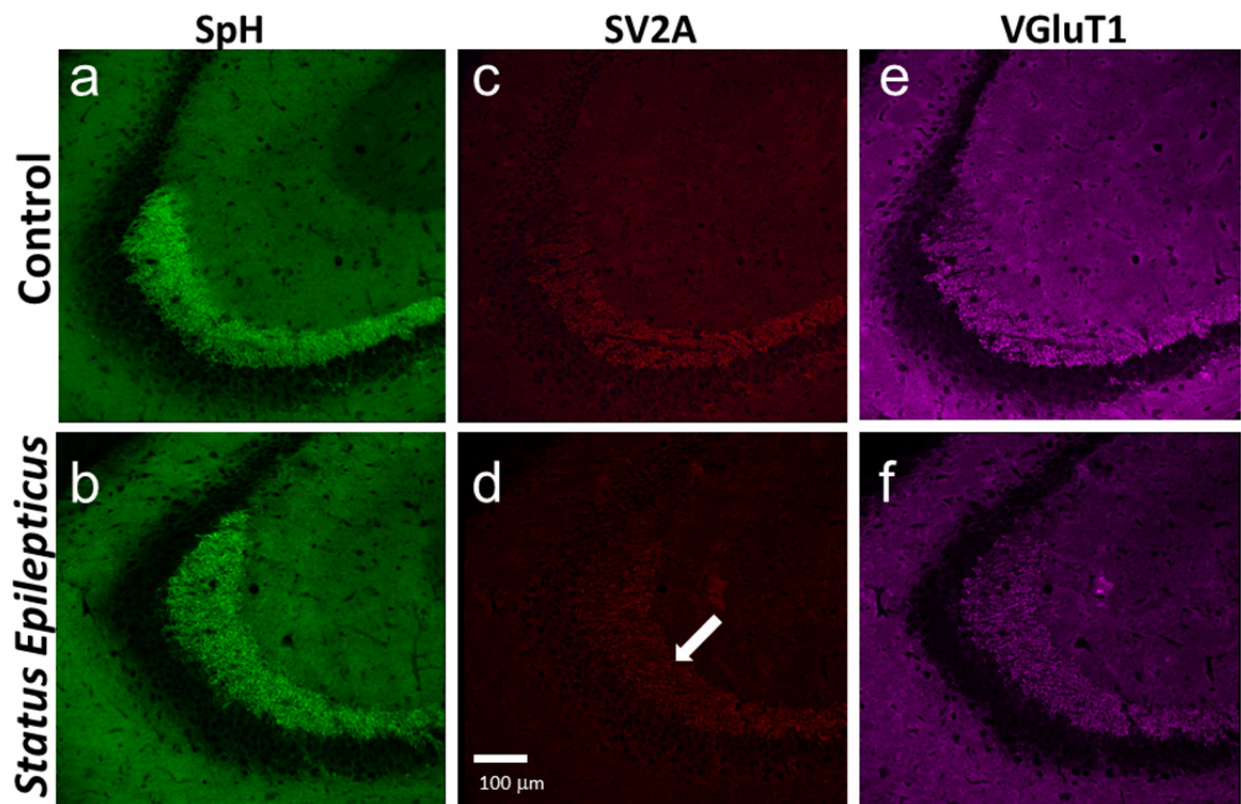


Figure 2. Immunostaining of SVA2 in transgenic SpH mice revealed a down-regulation of SV2A in the stratum lucidum (arrows) corresponding to the mossy fiber pathway in animal sacrificed 2 months after *status epilepticus*.

Figure 3

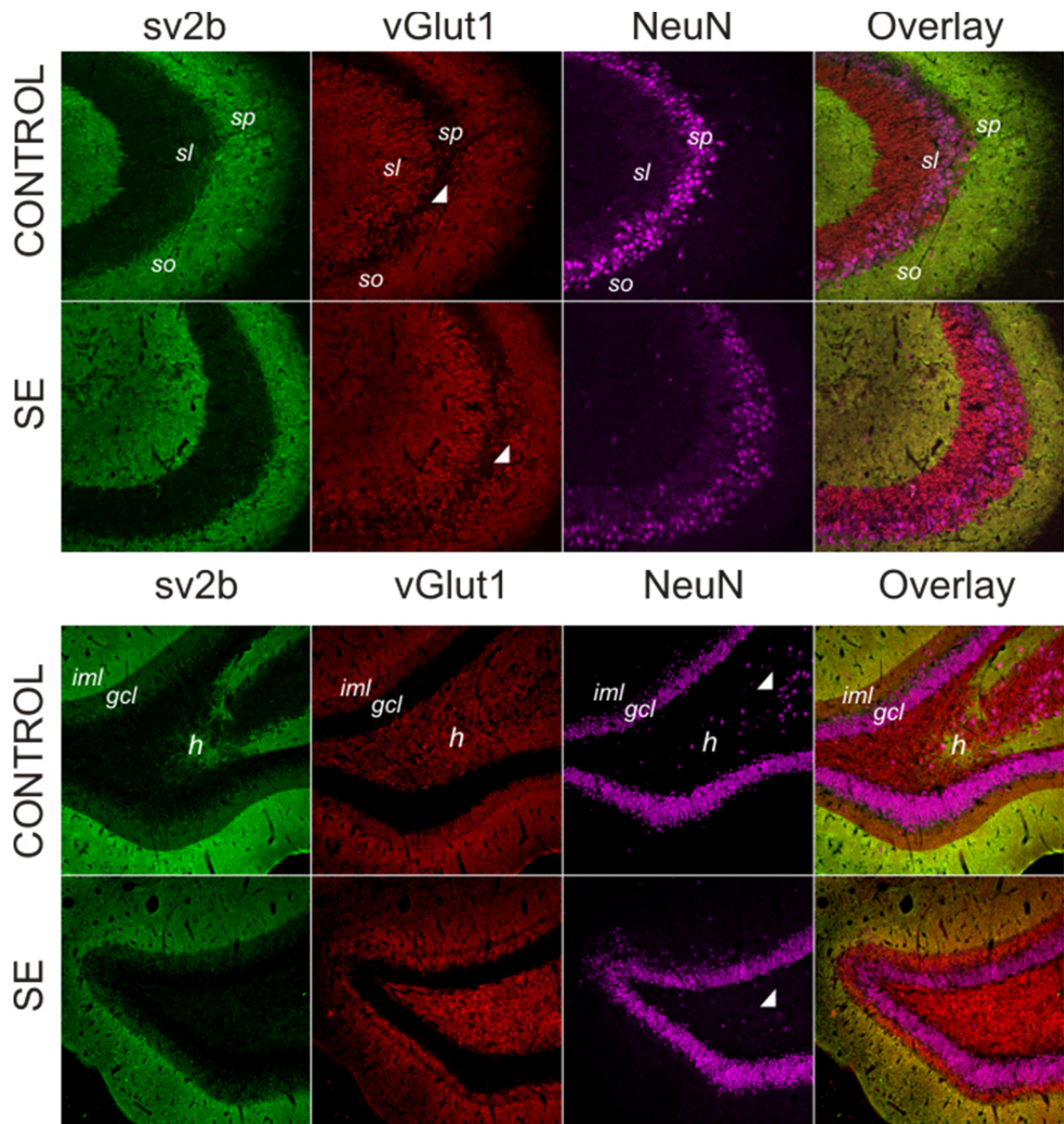


Figure 3. SV2B expression in hippocampus shows no qualitative differences after *status epilepticus*

Figure 4

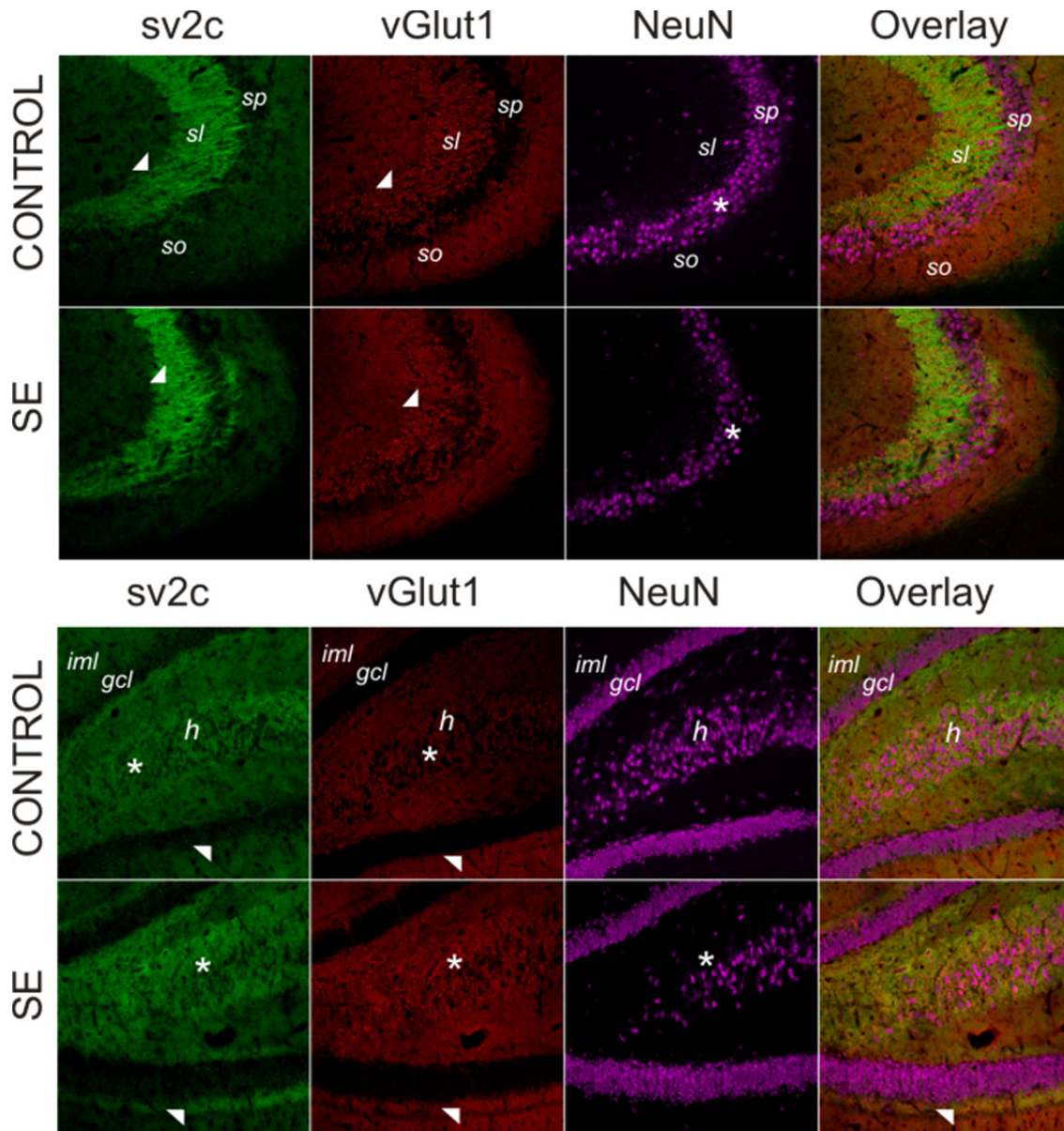


Figure 4. *Status epilepticus* (SE) induced up-regulation of SV2C in mice. Representative immunostaining experiments for SV2C, vGluT1 and NeuN in hippocampus of control and pilocarpine-treated mice that suffered *status epilepticus* (sacrificed 1 month after SE). Expression of Sv2c was increased in the mossy fiber pathway (arrows) in both panels. Top panel: Ca3 region, bottom panel: dentate gyrus. Please notice up-regulation of SV2C in the inner molecular layer of dentate gyrus just above granule cells. These Sv2c staining overlap(co-localized) to abnormally expressed vGluT1 proteins in same area indicating that SV2C is expressed in newly sprouted mossy fibers and boutons as part of the abnormal reorganization process that occurs after *status epilepticus*. This anomalous reorganization of excitatory (VGLuT1-expressing fibers) is proposed to play a major role in part in the pathogenesis of temporal lobe epilepsy.

3.2b. Chronic levetiracetam (LEV) treatment *in vivo* ameliorated abnormalities in presynaptic protein expression after *status epilepticus* in mice.

To determine whether chronic treatment with antiepileptic drug can modify (correct) abnormal expression of presynaptic proteins SV2A, SV2B and SV2C we used two different but complementary paradigms. A) Analysis of protein expression using western immunoblotting and B) Analysis of gene expression of SV2A, SV2B and SV2C using TaqMan-based real-time quantitative PCR. Control and experimental groups are similar as described for experiments of specific aim 3.1. Briefly, a) control SpH mice (*no status epilepticus*) injected with saline instead of levetiracetam (Control no treatment=C-NT), b) pilocarpine-treated mice that suffered *status epilepticus* injected with saline instead of levetiracetam (*status epilepticus*-no treatment=SE-NT), c) control SpH mice (*no status epilepticus*) that were injected with levetiracetam same days as post-*status epilepticus* mice (Control Treatment=C-T), and d) pilocarpine-treated mice that suffered *status epilepticus* and were injected with levetiracetam during one month 2 days after *status epilepticus* (*status epilepticus*-treatment=SE-T).

A) Analysis of protein expression of SV2, SV2B and SVC using western immunoblotting after *in vivo* treatment of epileptic mice with levetiracetam

Results: Data from these experiments revealed that *status epilepticus* induced a down-regulation of SV2A (25% reduction, but not significant at this time by Student t-test $p>0.05$, $n=5$), a no significant 24.1% increase in SV2B, and a statistically significant up-regulation of SV2C (52% increase, Student t-test, $p<0.05$, $n=5$) in animals that only received saline vehicle (0.9% NaCl) when compared to control saline-injected SpH mice group (**Figure 5A**) in a similar fashion as previously demonstrated in Sprague Dawley rats. Additional animals will be added to this data to improve power of the statistical analysis. After treatment with levetiracetam for 1 month (injections of drug 100mg/kg i.p in alternate days) the levels of SV2A significantly increased in animals that suffered *status epilepticus* (SE-T, $n=4$) (39.80% increased) when compared to levetiracetam-treated control group (C-T, $n=4$) (Student t-test, $p<0.05$) while expression of SV2B decreased 13.79% of control group ($p<0.05$). No significant change in the pattern of SV2C protein expression was observed detected (**Figure 5B**). These results indicated that chronic treatment with levetiracetam can revert or correct abnormalities of SV2A expression induced by *status epilepticus*, however this potentially therapeutic effect was not observed for SV2C which is abnormally increased after *status epilepticus*.

The significance of these findings are that treatment with levetiracetam may exert an antiepileptic action and neuroprotective effect by up-modulating expression of its own target SV2A, accordingly, levetiracetam-induced Sv2A up-regulation may explain why levetiracetam is selectively effective in chronically epileptic tissue with minimal effect in healthier non-epileptic brain in patients or animals models of epilepsy. It is known that levetiracetam failed to control convulsions/seizures in acute models of epilepsy while exerting a very potent antiepileptic effect in chronic models of epilepsy¹⁵⁻¹⁷.

Figure 5

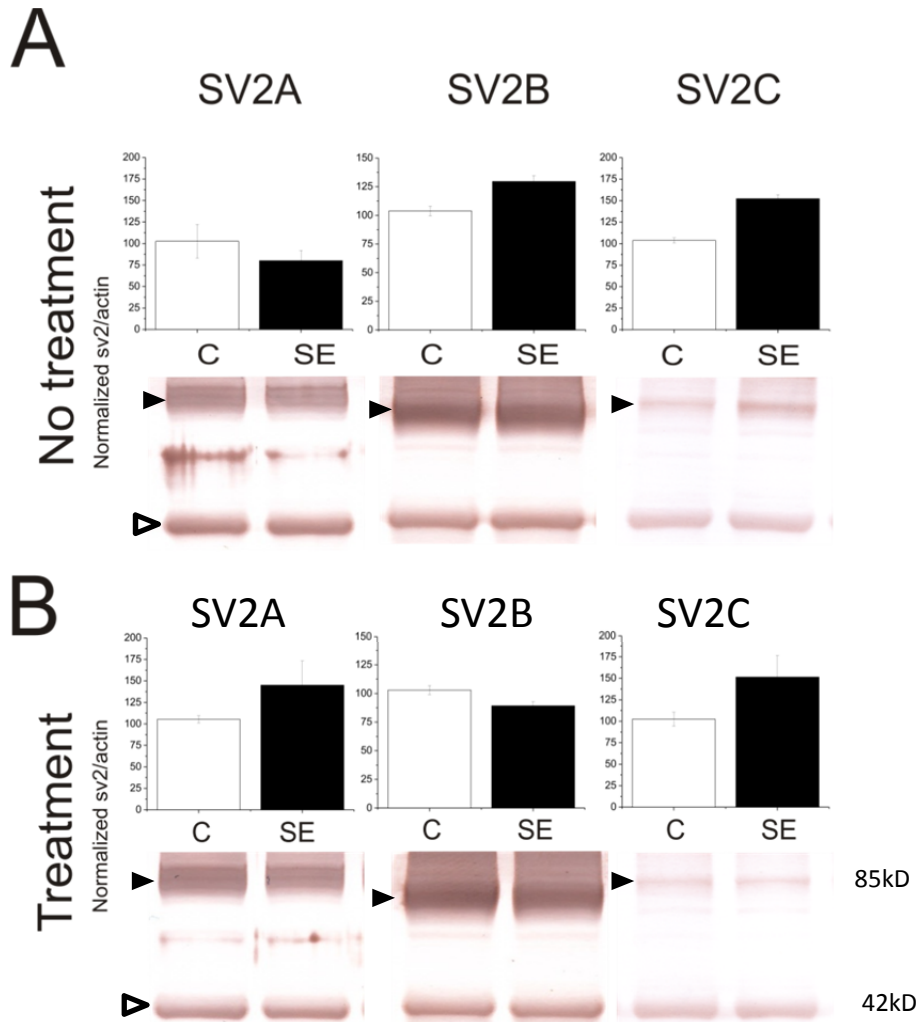


Figure 5. Effect of chronic in vivo treatment with levetiracetam (i.p) on the levels of protein expression of presynaptic vesicles SV2, SV2, and SV2C in hippocampus of SpH transgenic mice following the pilocarpine-induced *status epilepticus*. **A.** Graphs of normalized optical density for immunopositive bands show that *status epilepticus* (SE) induced a down-regulation of SV2A and up-regulation of SV2B and SV2C in no treatment groups (saline-injected). Measurements of optical density of immunopositive bands were normalized to expression of loading control β -actin (42kD) band. *Inset:* Representative immunoblottings for each of these SV proteins (black arrowheads, 85kD bands) and β -actin (white arrowhead, 42kD band). **B.** Chronic treatment with levetiracetam (100 μ g/Kg i.p) increased expression of SV2A (39.80% increase) and reduced expression of SV2B by 13.79% when compared to levetiracetam-treated control group (B). No significant change in the pattern the expression the SV2C was observed in the group treatment with levetiracetam.

B) Analysis of gene expression of SV2, SV2B and SVC using TaqMan-based real-time quantitative PCR after *in vivo* treatment of epileptic mice with levetiracetam.

In order to investigate changes in gene expression of these presynaptic proteins, we used the TagMan-based real time quantitative PCR technique using protocols already described in our previous studies^{4,7,18-20}. Experimental groups were described above. The rationale of this experimental design was to determine whether changes in proteins expression of SV2A, SV2B and SV2C are associated with corresponding changes in gene expression (mRNA transcripts) and to assess whether levetiracetam-induced SV2A up-regulation detected in the epileptic tissue was mediated by changes in gene expression machinery/process. For this purpose, one half (hemisphere) of the brain was used for protein expression analysis (western blotting) as described above while the other hemisphere was used for mRNA extraction. As internal controls we measure expression of calbindin D-28K (*calb1*) and vGluT1 (*Slc17a7*) which are down-regulated and up-regulated respectively in chronically epileptic tissue^{5,8}.

Isolation of mRNA and real-time quantitative PCR analysis of gene expression.

For isolation of total RNA, control and experimental groups (as described above) of SpH mice were anaesthetized and sacrificed 1 months (endpoint) after the beginning of injection sections (saline or levetiracetam). One hemisphere was used for protein isolation while the other hemisphere was used for total RNA isolation as previously reported^{4,7,18-20}. Briefly, tissue was collected, weighed (about 20 mg), homogenized, and processed for total RNA isolation at 4°C using the RNAqueous-4PCR Kit (Foster City, California, USA), following manufacturer's instructions. The concentration and purity of total RNA for each sample was determined by the Quant-iT RNA Assay Kit and [Q32857](#) Qubit fluorometer (Carlsbad, Invitrogen, California, USA) and confirmed by optical density measurements at 260 and 280nm using a BioMate 5 UV-visible spectrophotometer (Thermo Spectronic, Waltham, Massachusetts, USA). The integrity of the extracted RNA was confirmed by electrophoresis under denaturing condition. RNA samples from each set of control and epileptic rats were processed in parallel under the same conditions. RT were performed on an iCycler Thermal Cycler PCR System (Bio-Rad Laboratories, Hercules, California, USA), the High Capacity cDNA Reverse Transcription Kit (P/N: 4368814; Applied Biosystems, ABI, California, USA) for synthesis of single-stranded cDNA. The cDNA synthesis was carried out by following manufacturer's protocol using random primers for 1 µg of starting RNA. Each RT reaction contained 1000 ng of extracted total RNA template and RT reagents. The 20 µl reactions were incubated in the iCycler Thermocycler in thin-walled 0.2-µl PCR tubes for 10 min at 25°C, 120 min at 37°C, 5 s at 85°C, and then held at 4°C. The efficiency of the RT reaction and amount of input RNA template was determined by serial dilutions of input RNA. Each RNA concentration was reverse transcribed using the same RT reaction volume. The resulting cDNA template was subjected to quantitative real-time PCR (qPCR) real-time using Taqman-based Applied Biosystems gene expression assays, the TaqMan Fast Universal PCR Master Mix (ABI) and the StepOne Real-Time PCR System (ABI). Analysis of SV2A, SV2B and SV2C mRNA expression were carried out in a StepOne Real-Time PCR System using the validated TaqMan Gene Expression Assays for target genes. Gene expression analysis was performed using the TaqMan Gene Expression Assays Mm00486647_m1 for the target gene *Calb1* (RefSeq: NM_009788.4, amplicon size = 78 bp), Mm00812888_m1 for the target gene *Slc17a7* (RefSeq: NM_020309.3, amplicon size =

81 bp), Mm00491537_m1 for the target gene *Sv2a* (RefSeq: NM_022030.3, amplicon size = 79 bp), Mm00463805_m1 for the target gene *Sv2b* (RefSeq: NM_001109753.1, amplicon size = 88 bp), Mm01282622_m1 for the target gene *Sv2c* (RefSeq: NM_029210.1, amplicon size = 72 bp), and Mm99999915_g1 for the normalization gene glyceraldehyde-3-phosphate dehydrogenase *Gapdh* (RefSeq: NM_008084.2, amplicon size = 107 bp).

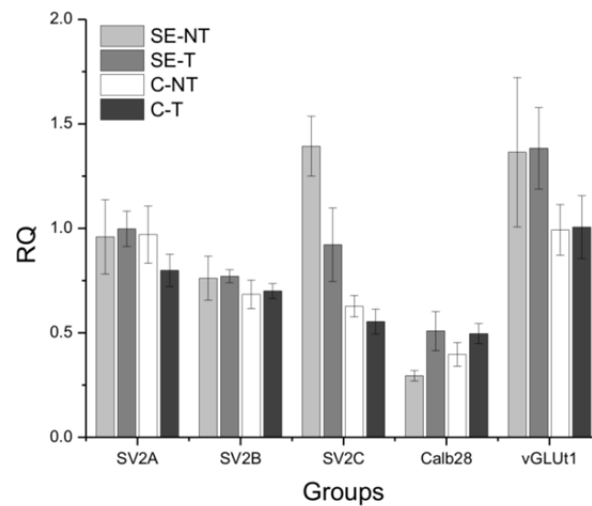
For qrtPCR analysis, each sample was run in triplicates. Each run included a no-template control to test for contamination of assay reagents. After a 94°C denaturation for 10 min, the reactions were cycled 40 times with a 94°C denaturation for 15s, and a 60°C annealing for 1 min. Three types of controls aimed at detecting genomic DNA contamination in the RNA sample or during the RT or qrtPCR reactions were always included: a RT mixture without reverse transcriptase, a RT mixture including the enzyme but no RNA, negative control (reaction mixture without cDNA template). The data were collected and analyzed using OneStep Software (ABI). Relative quantification was performed using the comparative threshold (CT) method after determining the CT values for reference (*Gapdh*) and target (*sv2a*, *sv2b* or *sv2c*, *calb28* and *Slc17a7*) genes in each sample sets according to the $2^{-\Delta\Delta C_t}$ method ($\Delta\Delta C_t$, delta-delta CT)^{21,22} as described by the manufacturer (ABI; User Bulletin 2). Changes in mRNA expression level were calculated after normalization to *Gapdh*. As calibrator sample we used cDNA from arbitrarily selected control rat. The $\Delta\Delta C_t$ method provides a relative quantification ratio according to calibrator that allows statistical comparisons of gene expression among samples. Values of fold changes in the control sample versus the post-SE samples represent averages from triplicate measurements. Changes in gene expression were reported as fold changes relative to controls. Data were analyzed by analysis of variance (ANOVA) (followed by post-hoc analysis) or via paired *t*-test to check for statistically significant differences among the groups (significance *P* value was set at <0.05).

Results: Our data indicate a significance change in **sv2a gene expression** among the different groups (ANOVA, $p < 0.05$, **Figure 6**). Post-hoc analysis revealed that *in vivo* levetiracetam treatment reduced the expression of *sv2a* transcripts in control group (no *status epilepticus*) when compared to all the other groups ($p < 0.05$). This was unexpected considering that in protein expression analysis this effect was not observed. In addition, unlike effect of chronic treatment with levetiracetam SV2A protein expression, *sv2a* transcripts were not upregulated in levetiracetam-treated *status epilepticus* group. Hence, levetiracetam-induced SV2A up-regulation may be mediated by non-transcriptional mechanisms, but perhaps by slowing down SV2A protein degradation, etc. No significant changes were detected in **sv2b gene expression** among the groups. However, a significant change was observed for **sv2c gene expression** (ANOVA, $p > 0.0001$, $F = 11.87$) where *status epilepticus* induced a significant *sv2c* upregulation (SE-NT) when compared to saline-injected control group (C-NT) (post-hoc Tukey Honest, $p < 0.01$). Interestingly, in contrast to SV2C protein expression, chronic treatment with levetiracetam induced a significant 33.8% and 11.7% reduction of *sv2c* levels in treated *status epilepticus* and control groups respectively when compared to saline-injected *status epilepticus* and control groups. These data indicate that levetiracetam treatment can reduce abnormally increased *sv2c* transcripts after *status epilepticus*. Changes were detected among groups for calbindin 28 expression. *Status epilepticus* induced a down-regulation of calbindin 28 (*calb28*, *calb1*) and chronic treatment with levetiracetam corrected (increased) expression of these transcripts compared

to control levels (post hoc Tukey Honest, $p < 0.05$). Significant changes were noticed among groups for expression of *Slc17a7* gene (*vGLUt1*). *Status epilepticus* induced a significant upregulation of VGLuT1 when compared to control (Tukey Honest, $p < 0.01$) that was not further modified after treatment. VGLuT1 increased in both levetiracetam and saline-injected groups after *status epilepticus*.

The relevance of these findings is unclear as SV2C plays an unknown role in transmission release or in the pathogenesis of epilepsy. Our data indicate that SV2C may be also involved in the antiepileptic action of levetiracetam in chronically epileptic brain. However, there is a differential effect of seizures on SV2A and SV2C (down- and up-regulation respectively) while effect of levetiracetam partially restore these abnormalities.

Figure 6



SE-NT *Status epilepticus* non treated group (saline injections)
 CN-T Control non-treated group (saline injections)

SE-T *Status epilepticus* levetiracetam-treated group
 CT-T Control levetiracetam-treated group

Figure 6. *Changes in gene expression after levetiracetam in vivo treatment in different groups of SpH mice.* Bar graphs represent percentage changes of triplicated results \pm SE, and adjusted to the *Gapdh* gene (normalizing gene, housekeeping gene). Data was obtained using the TaqMan-based real-time quantitative PCR and delta-delta CT analysis. Levetiracetam treatment did not change expression of *sv2a* in epileptic brain but reduced transcripts in control group. No significant changes were detected for *sv2b* expression. *Sv2c* expression was up-regulated after *status epilepticus* but chronic treatment with levetiracetam induced a reduction of *sv2c* expression, although *sv2c* levels remained above controls. Changes were detected among groups for calbindin 28 expression. *Status epilepticus* induced a down-regulation of calbidin 28 (*calb28*) and levetiracetam corrected (increased) expression of these transcripts compared to control levels (Post-hoc Tukey Honest, $p < 0.05$). Significant changes were noticed among groups for expression of *Slc17a7* gene (*vGLUt1*). *Status epilepticus* induced a significant upregulation of vGLU1 (*Slc17a7* gene) when compared to control (Post-hoc Tukey Honest, $p < 0.01$) that was not further modified after treatment.

Milestones: The following milestones were accomplished during year 3 (2013-2014).

- (a) We have shown that chronic treatment of animals with levetiracetam is effective in inhibiting abnormally enhanced presynaptic vesicle release of in mossy fibers of granule cells after *status epilepticus*.
- (b) In addition, we found that chronic treatment with levetiracetam increased protein expression of SV2A (levetiracetam targets) in mossy fiber pathway while reducing seizure-induced increases in SV2C (gene expression of sv2c by qrtPCR) in hippocampus after pilocarpine-induced *status epilepticus*.
- (c) Our data indicate that levetiracetam is effective in counteracting pro-epileptogenic changes in transmitter release and presynaptic molecules if administered after the epileptogenic insult (*status epilepticus*).

REPORTABLE OUTCOMES:

National Meetings

Data from electrophysiological and pharmacological experiments were presented in 1 scientific meeting in 2013 (1-2) and new data will be presented in another two meetings in 2014 (3-4).

1) *Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats.* **E. G. Sanabria**, L. Pacheco, J. Zavaleta, F. Shriver, L. M. Rambo, C. Upreti, **P. K. Stanton**. Society for Neuroscience Meeting in San Diego, CA, Nov 9-13, 2013

2) *Synaptic Vesicle Protein Expression in Sprague-Dawley Rats Treated with the Pilocarpine Model for Mesial Temporal Lobe Epilepsy.* Mayra Velazquez, L.F. Pacheco, **E. R. Garrido-Sanabria**. 25th Anniversary Conference, HENAAC Conference, October 3 - 5, 2013, New Orleans.

3) *Chronic treatment with levetiracetam upregulate SV2A and reduce abnormally augmented presynaptic vesicular release after pilocarpine-induced status epilepticus.* Luis F. Pacheco, Vinicius Funck, Nuri Ruvalcaba, Jose M. Rodriguez, Daniela Taylor, Rubi Garcia, Jason Zavaleta, Chirag Upreti, **Patric K. Stanton**, **Emilio R. Garrido-Sanabria**, the Military Health System Research Symposium, to be held on August 18-21, 2014.

4) *Treatment with levetiracetam ameliorates abnormal presynaptic vesicular release and altered presynaptic protein expression in a glutamatergic pathway after pilocarpine-induced status epilepticus.* **Emilio R. Garrido-Sanabria**, Luis F. Pacheco, Vinicius Funck, Nuri Ruvalcaba, Jose M. Rodriguez, Daniela Taylor, Rubi Garcia, Jason Zavaleta, Chirag Upreti, **Patric K. Stanton**. This abstract will be submitted to the 68th Annual Meeting of the American Epilepsy Society, Dec 5-9, 2014, Seattle, Washington.

CONCLUSIONS:

Our results indicate that chronic treatment with the antiepileptic drug levetiracetam after *status epilepticus* can reduce abnormally enhanced presynaptic vesicle release of glutamatergic mossy fiber boutons in pilocarpine-treated SpH transgenic mice. In a previous study we reported a long-lasting abnormality of presynaptic structure and function in experimental epilepsy². Specifically, multiphoton microscopy was used to directly measure prospective changes in vesicular recycling properties from hippocampal mossy fiber presynaptic boutons in control versus pilocarpine-treated chronic epileptic SpH21 transgenic mice expressing SpH preferentially at glutamatergic synapses. We detected significant increases in action potential-driven vesicular release at 1-2 months after pilocarpine-induced *status epilepticus*. Results from Year 3 of this proposal also indicate that levetiracetam may decrease exaggerated presynaptic vesicular release by affecting the presynaptic release machinery at excitatory pathways. Specifically, chronic treatment with levetiracetam increased deficient expression of SV2A in hippocampus (SV2A is a molecular target for levetiracetam) and reduced the abnormally increased levels of SV2C in similar pathway. Additional experiments are in progress to detect changes in another presynaptic protein mGluR2. Deciphering the mechanisms of levetiracetam-induced changes in presynaptic proteins may lead to a novel antiepileptic mechanism for this antiepileptic drug. In addition, additional studies are necessary to develop and assess the role of new antiepileptic drugs that target newly reported changes in presynaptic proteins, specifically SV2C.

Significance: These data indicate that presynaptically acting drugs such as levetiracetam reduces hyperexcitability and inhibit presynaptic transmission in mesial temporal lobe epilepsy. Preventive treatment with antiepileptic drug is a feasible strategy to reduce the development of epilepsy (specifically post-traumatic epilepsy). In our study, we investigate whether treatment with the drug levetiracetam immediately after pilocarpine-induced status epilepticus (epileptogenic insult) significantly reduced abnormally enhanced presynaptic vesicle release and reduce molecular abnormalities in presynaptic molecules (i.e. SV2A, SV2B, SV2C) in treated epileptic mice. Interestingly, we also detected that *status epilepticus* induced up-regulation of SV2C while producing down-regulation of SV2A in the mossy fiber pathways. In vivo treatment with levetiracetam partially corrected both protein and mRNA gene expression changes in SV2C while only partially restored deficient SV2A protein levels after *status epilepticus* indicating that SV2C may also play a role in the epileptogenic process. LEV is a novel antiepileptic drug that binds to presynaptic targets SV2A. Although SV2A has been identified as the main molecular target for levetiracetam^{17,23,24}, our data indicate that “pro-epileptic” changes in SV2C may become a novel antiepileptic therapeutic target for new pharmacological drugs in order to modify presynaptic transmitter release and reduce hyperexcitability in epilepsy.

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